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Biophysics Research Laboratory of the Department of Medicine
Harvard Medical School and
Peter Bent Brigham Hospital

OFFICE OF NAVAL RESEARCH

A Final Report on the Activities of the Biophysics Research Laboratory under Contract NR 119-377 - Nonr 1866(04)

October 8, 1957

Submitted by: Bert L. Vallee, M.D.
Scientific Director

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The Biophysics Research Laboratory of the Department of Medicine, Harvard Medical School and the Peter Bent Brigham Hospital was dedicated on May 17, 1954. The laboratory, which comprises an area of about 7,500 square feet, was completely renovated within the existing facilities of the hospital. It has been fully equipped with all the instruments necessary for investigation in emission and absorption spectroscopy, intermediary metabolism and physical chemistry. It was established with major grants from the Rockefeller Foundation and the National Institutes of Health. Additional funds were received from private donors and from Harvard Medical School. The first application to the Office of Naval Research was submitted in April 1952 and a contract went into effect in 1953. Up to the time of the dedication of the laboratory at the Peter Bent Brigham Hospital, the work was carried out in the Department of Biology and in the Spectroscopy Laboratory at the Massachusetts Institute of Technology.

The present report constitutes a review of our activities under this joint grant from the Navy, Army and Air Force which was administered by the Office of Naval Research.

Scientific, philosophical and educational considerations were fundamental in the establishment of the Biophysics Research Laboratory:

1. The laboratory was to be concerned primarily with fundamental investigation. Biophysics, physics, physical biochemistry, and quantitative biology were to be emphasized. It was anticipated that the existence of medical facilities would occasion extensions into the clinic

2. It was envisioned that the study of the function of metals in biology in general, and catalytic phenomena in particular would become a primary target

of the scientific effort. The development of quantitative spectrographic methods opened new avenues for a concentrated effort in this field which had not previously been approached systematically. The elucidation of the molecular basis of metalloenzyme action appeared especially promising and was to constitute a specific objective.

Continued fundamental studies in emission and absorption spectrography were considered essential to a proper evaluation of such biological interests.

3. The establishment of the laboratory in a hospital of relatively small size was expected to result in closer contact between scientists and the physicians of the hospital. A reorientation of some phases of medical teaching to pre- and postdoctoral students of medicine and of the sciences was anticipated to be a desirable result of such an association. It was hoped that the laboratory would attract individuals from both medicine and the various scientific disciplines.

It is a pleasure to report that these major objectives have been achieved.

This report will emphasize the highlights of these past years' work. Much of it has been published in the scientific literature and copies have been sent to the Armed Forces. A full bibliography is attached.

I. SPECTROSCOPY

A. Porous Cup Electrode Sparking Method: The porous cup electrode technique, repeatedly described in our annual reports, has been standardized and has remained the routine spectrographic technique of the laboratory. It has not required nor undergone any significant changes in the last year and a half. Details of the method, such as internal standardization, control of ashing temperature, plate calibration and densitometry, have been clarified to a high degree. The experience of the last three years has now reassured us that under present working conditions this method is probably as adequate as any that can be designed for biological emission spectroscopy without major alterations in existent instrumentation. Compilation of data on reproducibility and precision indicate that 5 to 7 per cent is a reasonable estimate of the standard deviation at any level of concentration from .01 ppm to 500 ppm. We feel that sufficient experience with the method and its performance has now been gained to warrant placing it on record in the scientific literature and therefore several publications are planned. A summary chapter in "Methods of Biochemical Analysis", Volume 3, is contemplated.

B. Noble Gases and the DC Arc: The study of the effects of helium, argon, neon and krypton on the DC arc, begun at Massachusetts Institute of Technology, was completed. Early findings of an increased signal to noise ratio and selective enhancement of certain spectral lines were confirmed and extended. A general theory was formulated which adequately predicts and explains the observations. The selective enhancement is apparently due to a resonance phenomenon involving collisions of the second kind between metastable gas atoms and those of the elements to be excited. When the transition of the atom is within the energy range of the excited gas atom, greater efficiency of excitation

for these transitions results. These findings are reported in detail in several publications: (J. Opt. Soc. Am. 46: 83, 1956, J. Opt. Soc. Am. 46: 138, 1956; J. Opt. Soc. Am. 46: 77, 1956).

C. The Direct Reading Flame Spectrometer: A direct reading flame spectrometer for the simultaneous analysis of sodium, potassium, calcium and magnesium has been completed and is in routine use in the laboratory. With this instrument it is possible to determine all four elements simultaneously in one sample. The limits of detection are: sodium .01, calcium .1, potassium .3 and magnesium 1 ppm. Thus far, no effort has been made to extend these limits. The Jarrell-Ash Company, Newtonville, Massachusetts, is under contract to manufacture this instrument, but a commercial model has not yet appeared on the market in spite of heavy demand. The subject matter pertaining to these studies is reported in three articles: (Anal. Chem. 28: 176, 1956, Anal. Chem. 28: 180, 1956; Anal. Chem. 28: 1066, 1956). The general subject of flame photometry has also been reviewed in a chapter in "Methods of Biochemical Analysis", Volume 3, 1956. In addition, a special technical report to the Office of Naval Research was published by the Department of Commerce as "Direct Reading Flamespectrometry. Principles and Instrumentation" (PB 111743), 1956.

D. The Cyanogen-Oxygen Flame: The study of the direct reading procedures with the hydrogen-oxygen flame persuaded us that the extension of emission spectrographic work in biology should not be sought in arc or spark spectroscopy. It seemed that a flame could constitute the "ideal" source, if a particular fuel could be found which would energize the sample sufficiently to excite transitions of atoms having a higher excitation potential than those characteristic of alkali metals and alkaline earths. No such flame was in

existence for spectrographic purposes. We were able to utilize the reaction $C_2H_2 + O_2 \rightarrow H_2 + 2CO + E$ for this purpose and to demonstrate that spectrometry of the transition metals could be performed (J. Opt. Soc. Am. 46: 77, 1956). This is the first really new flame source discovered in more than 50 years. Further quantitative measurement by photometric means was feasible (Anal. Chem. 28: 1783, 1956). Our studies of the four channel flame spectrometer indicated the practicability of substituting a direct reading procedure for photographic recording; hence, a "direct reading acetylene-oxygen flame spectrometer" has now been developed which presently allows the analysis of copper, manganese and magnesium. An extension to other transition elements is contemplated.

The sensitivity of detection of these three elements is 50 parts per billion. The total volume of solution, .04 cc./15 sec., represents a major step forward in lowering the limit of detection and decreasing the volume of solution necessary for analysis. In the course of these studies various physical factors effecting the temperature of the flame have been explored. It has been shown that a high flow rate of an aqueous sample materially cools the flame and decreases sensitivity. Fundamental processes accounting for this circumstance have been investigated and a mathematical formulation predicts the events adequately. These investigations are in preparation for publication.

5. The Coenzymeter: As a result of our studies in enzymology it became apparent that a spectrometer capable of measuring reactions at 340 mμ, the wavelength at which DPNH absorbs maximally, would be extremely useful, both for research investigators and for clinicians now interested in adapting DPNH dependent reactions to clinical diagnosis as in lactic dehydrogenase and transaminase determinations. A simple instrument was therefore designed which is now in commercial production. It has been given the tradename "Coenzymeter".

The instrument has been acclaimed by hospitals and practitioners, since it makes unnecessary an expensive ultraviolet spectrophotometer selling at 15 times the price of this instrument. Details of design and construction are in preparation for publication.

II. ENZYMOLOGY

A. Metalloenzymes. The study of metalloenzymes is the major biochemical interest of the laboratory; investigations in this field have led to the discovery of zinc in five enzymes not hitherto known to contain this metal: the ADH of yeast, ADH of equine liver, GDH of bovine liver, LDH of rabbit muscle and carboxypeptidase of bovine pancreatic juice. In all instances, zinc has been found to be an intrinsic part of the enzyme molecule and essential to its catalytic behaviour. These inferences have been possible by virtue of compositional, structural and functional studies in which instrumental analyses involving spectroscopy, ultracentrifugation and electrophoresis have been employed. The functional properties have been tested further by the use of metal binding inhibitors and the study of the kinetics of their interactions with these enzymes. Another group of pyridine nucleotide dependent dehydrogenases (PND) have also been shown most likely to be metalloenzymes, they have been referred to as pyridine nucleotide metallodehydrogenases (PND). This work has been reported in various publications: (Proc. Nat. Acad. Sci. 41: 327, 1955. Chapter in "Advances in Protein Chemistry", Volume 10, 1955; J. Biol. Chem. 221: 491, 1956. J. Am. Chem. Soc. 77: 5196, 1955; J. Biol. Chem. 217: 253, 1955, J. Am. Chem. Soc. 78: 1771, 1956; J. Am. Chem. Soc. 78: 5879, 1956; J. Biol. Chem. 225: 185, 1957). These studies have, for the first time, implicated a metal to be involved

in enzymatic dehydrogenation reactions. The finding of zinc in carboxypeptidase has reconciled previously discrepant reports in the literature. A further review requested by the Editor, will appear in "The Enzymes", Volume I, Part II, 2nd Edition.

B. Kinetics: Extensive kinetic studies have resulted in a model for the action of a metal chelating agent, 1,10 phenanthroline (OP) in bringing about inhibition. Three papers on this subject are in preparation for publication.

C. Mixed Complexes. The interaction of 1,10 phenanthroline with LADH has been studied in detail. It has been possible to show that this agent exerts its effect by binding directly to the zinc of the enzyme protein, forming a ternary complex in situ. The studies were reported at the meetings of the Federated Societies of Biology (Fed. Proc. 16: No. 1129, 1957) and details currently are in press in the J. Am. Chem. Soc.

D. Other Active Groups: Since sulfhydryl groups had been stated by other investigators to be active sites in these enzymes which have now been identified as zinc metalloenzymes, studies were undertaken on -SH groups. These groups do not bind the zinc in YADH, nor do they seem to bind substrate or coenzyme. SH groups do appear to be involved in the maintenance of the structure of the active enzyme. The general interaction of -SH binding reagents with proteins has also been studied. A preliminary report has been rendered (Fed. Proc. 16 No. 1530, 1957). Three papers on these findings are in preparation.

E. Cadmium Protein. We have succeeded in isolating a cadmium protein from equine kidney cortex. It contains 1.7% of the metal/gram dry weight of protein. This is the first instance of the isolation of a natural product containing cadmium, an element which has not been thought to have biological significance. Studies are currently underway to ascertain the specific nature and

function of this unusual metalloprotein. A preliminary report has been published in the J Am. Chem. Soc. 79: 4813, 1957.

F Electron Transport in Mitochondria: An electron transport system in mitochondria, reducing triphenyltetrazolium chloride (TTZ) to triphenylformazan, was found to be activated by ATP and further by magnesium, manganese and nickel ions. Maximum activation was observed when the metals were equimolar to ATP. It was postulated that a metal-ATP complex is the true activator (Nature 176: 286, 1955). Further factorial studies (unpublished) have shown critical interactions between different metals modifying this effect. A paper on this subject is in preparation for publication.

G Metal Content of Subcellular Fractions: The above investigations demanded extension by metal analysis of subcellular fractions. Connective tissue and all subcellular fractions of rat liver studied (nuclei, mitochondria, microsomes and clear supernatant fluid) were shown to contain metals in substantial quantities and in characteristic patterns of distribution, giving a new and objective base line for the interpretation of experiments with these important constituents of cells (J. Biol. Chem. 226: 911, 1957). The induction of magnesium deficiency in rats caused significant dislocations not only of magnesium in connective tissue and mitochondria but also of calcium and iron, changes which could not have been discerned had liver tissue alone been analyzed. (Proc of the First Conf. on Biophysics, Columbus, in press). These studies are currently being extended.

H. Hibernation: Investigations were carried out on the biochemical, cellular changes incident to cold adaptation and on hibernation. Semster liver mitochondria from animals either hibernating or exposed to cold environments showed markedly increased succinic oxidase, succinic dehydrogenase - cytochrome

C reductase, and cytochrome oxidase activities. Since oxidative phosphorylation was normal, or even high, the oxidative efficiency of these animals was much increased. Rats, a non-hibernating species, showed no such changes in their adaptation to cold. This work was the basis of an accepted thesis for the Ph.D. degree (by E.R. Chaffee) and is being prepared for publication.

III. CLINICAL INVESTIGATION

A. Copper in Myocardial Infarction: Early studies had shown that serum copper is markedly increased subsequent to myocardial infarction. It was shown that this phenomenon is accompanied by an increase in paraphenylenediamine (PPD) and benzidene oxidation, allowing the assumption that the rise of serum copper is the result of an increase in ceruloplasmin, the copper bearing protein of human serum which exhibits enzymatic activity toward these two substrates. The increase in benzidene oxidation was greater than could be accounted for by the increase in copper alone, suggesting the appearance of an additional enzyme capable of oxidizing benzidene (New Eng. J. Med. 255: 105, 1956).

B. Lactic and Malic Dehydrogenase in Myocardial Infarction: Our studies of these pyridine nucleotide dependent metallodehydrogenases (see II A) led to their investigation in myocardial infarction. Both enzymes are significantly increased in this condition and allow simple, accurate and rapid diagnostic procedures. The commercial (see I. E) was the result of these studies which have been reported in the New Eng. J. Med. 255: 449. 1956. This method has been used as a routine diagnostic procedure at this hospital and some 500 patients have been studied to date; their records are undergoing analysis at this moment.

C. Magnesium and Acute Renal Failure. The multichannel flame spectrometer was adapted for the measurement of magnesium in serum. It has been

shown that this element is significantly increased in acute renal failure resulting from a variety of causes. The hypermagnesemia contributes greatly to the symptomatology of these individuals and explains clinical manifestations on the bases previously had been obscure. The elevation in serum magnesium roughly follows that of potassium and returns to normal with restoration of kidney function. These findings are in press in the New Eng. J Med.

D. Zinc in Cirrhosis: Our studies in post-alcoholic cirrhosis led to an exploration of the possible mechanism leading to the establishment of this disease. Serum zinc concentrations were found significantly decreased (New Eng. J. Med. 255: 403, 1956). These studies have now been extended and currently indicate severe metabolic abnormalities which may be summarized as consisting of a decrease in serum zinc, proportionate to the severity of the disease, marked zincuria and marked decrease of liver zinc concentrations. In a series of clinical studies oral zinc sulfate caused a cessation of zincuria and a return of liver function as evidenced by changes in BSP retention. A chemical mechanism to explain these changes has been hypothesized. A preliminary account has been published (J.Clin. Invest. 36 933, 1957). This material is in press in the New Eng. J Med - November 28, 1957

IV MATHEMATICS

A. The Sequential Probability Ratio Test (SPRT): The clinical studies just reported have led to the development of the sequential probability ratio test as a design for clinical experiments (New Eng J. Med. 256: 498 1957). This novel statistical design allows the economical and efficient plan of clinical experiments, terminating them at the very moment a given biochemical, physiological or pathological hypothesis has been validated or rejected. This procedure eliminates a large fraction of previous uncertainty, concerning both the adequacy of data

obtained and the sufficient number of observations that need to be made in order to arrive at a valid conclusion. Several applications have been made (New Eng. J Med. 265: 403, 1958; New Eng. J Med 255: 449, 1956).

B. Stochastic Models: Observations in chemical kinetics have to be made sequentially in time; it is commonly assumed that if all observational methods could be made without error, the observations at any given time would reflect the true state of a given reaction at this particular juncture. Irregular fluctuations in the data of well controlled reactions would suggest that certainty in chemical kinetics is no greater than in physics and biology.

Stochastic models have been devised as mathematical constructs for a kinetic theory which would allow the inclusion of a probabilistic measure of the uncertainty in the interpretation of kinetic data. The models do not substitute for, but extend past, deterministic theories. The theory has been set up for unimolecular and bimolecular reactions, and equations have been solved for these systems. A general theory for multimolecular processes has also been developed but several partial differential equations thus far resist solution. This study was the subject of a Ph.D. thesis entitled "A Stochastic Approach to Reaction Kinetics" by A.F. Bartholomay

V. ANALYTICAL CHEMISTRY

Aside from our spectrographic methods, a variety of others have been investigated.

A. Determination of Zinc (by Means of Methyl Cellosolve and Dithizone): Conventional analytical procedures for zinc call for phase separations, requiring the extraction of the element from an aqueous solution into a carbon tetrachloride or chloroform phase by means of dithizone. Such separation procedures are always time consuming, difficult and compare unfavorably with known monophasic procedures.

By dissolving this reagent in methyl cellosolve, which is miscible with water, a monophase method has been devised which, in terms of precision, accuracy and sensitivity, compares very favorably with the conventional extraction methods. (Anal. Chem. 26: 914, 1954).

B. Zinc in Urine: A new method of zinc analysis in urine has been devised by the simple expedient of extracting urine with dithizone as if it were a simple aqueous solution of inorganic ions, an assumption which has been justified by the results obtained with this method. Time-consuming and tedious ashing procedures are thus avoided with a concomitant improvement of precision as a result of this simplification. The principle of this method should be applicable to the determination of many other metals in urine. (This material is in preparation for publication).

C. Concentration Techniques: Increase of sensitivity of determination of trace elements might be expected by concentration methods. Zinc, iron, copper and cobalt have been separated from biological material by ion exchange procedures resulting in an aggregation of these elements and allowing their precise determination by emission spectrography. A preliminary report has been published (Anal. Chem. 27: 315, 1955). A detailed account of these studies is in preparation for publication.

D. Contamination and Separation: The general interest of the laboratory required a thorough appraisal of contamination hazards. The significance of metal contamination in work of the type here described can best be compared to the need for sterility in bacteriology and virology. The knowledge and viewpoints and experiences of this laboratory have recently been summarized in a chapter entitled "Separation and Concentration of Millimicrogram Quantities of Metals and Contamination Hazards" in "Methods of Biochemical Analysis", Volume 3, 1957.

VI. EDUCATIONAL ACTIVITIES

The educational objectives of the laboratory have received increasing attention during the past year and a half. While their ultimate range cannot be foreseen, their scope has become apparent.

A. Pre- and Postdoctoral Training. Fifteen young scientists have thus far received training in the laboratory, nine are continuing in residence. The diverse educational backgrounds of these gentlemen testifies to the achievement of the stated aims. Opportunities of contact for scholars educated in different though related disciplines: physics, biophysics, chemistry, biochemistry, mathematics, and medicine. There is little doubt that this very diversity of backgrounds has resulted in increasingly profitable exchange of ideas. The changing intellectual viewpoints and mode of procedure of all concerned can be traced readily to such mutual influences. The adaptation of these individuals to each others' different intellectual viewpoints has been one of the most stimulating features of the laboratory's activity, an aspect which has been encouraged wherever possible. It cannot be stated with certainty to what degree this elusive characteristic of the laboratory's operation contributed to achievement, but all participants are convinced that it plays a major and most significant role.

Formal and informal course work was designed to remove barriers of communication and thought. In particular, a brief course in human biology, which constitutes a deviation from tradition, should be mentioned. On request, a member of the staff, trained primarily in medicine, undertook to teach some aspects of this subject to those members of the laboratory whose primary training was in the natural sciences. The course was voluntary and was taught on an informal basis. It was received enthusiastically and achieved the desired objective: improved

effectiveness of the staff's interaction and integration with the educational environment.

The laboratory is in a somewhat anomalous educational position. It harbors individuals aiming primarily at further education and training in the biological sciences, but in an environment which is preoccupied with the practice and teaching of medicine. Thus no formal, concrete program for the recruitment of graduate students has existed.

It was possible, nevertheless, to make arrangements with the Harvard School of Public Health, the Biology Department of Harvard University, the Department of Biology of the Massachusetts Institute of Technology and the University of London to allow five candidates to undertake their thesis work in the Biophysics Research Laboratory. Three of these have now received their doctorate degrees in mathematical biology, biology and biophysics, respectively. The two remaining candidates have just begun their work.

Five of the research fellows hail from overseas: two from Japan, one from Switzerland; one from Great Britain; one from Paraguay.

B. Medical School Teaching and Hospital Activities: The staff of the laboratory has had increasingly profitable, frequent and pleasant contact with the physicians and other research laboratories of the hospital. The evolution of these relationships has been left to chance and interest in the belief that integration will be achieved most effectively without pressure or editorial direction.

In 1954 an experimental teaching program was established to investigate the feasibility of integrating biochemical approaches to internal medicine within conventional medical teaching but without a change in curriculum. Two groups of medical students have now been processed through the courses in second and third

year medicine given by the laboratory staff, and a third group is due to arrive in November. The enthusiastic reception of the course by the students may be an indication of the outcome of this program which cannot be evaluated finally until further data have been accumulated. Several of these students have asked for and received permission for elective work in the laboratory

Weekly teaching "rounds" for the house staff and fourth year students have been instituted. These "rounds" again stress the relationship of dynamic and physical biochemistry to the basis of medical thinking and practice and attempt to delineate those facets of the sciences which may now serve as a basis for generalization and application.

Doctors Valles, Koch and Wacker have been appointed Tutors in Medical Science for the freshman medical school class. Dr. Price has been appointed a Tutor in Biochemistry in the tutorial program of Harvard College. Dr. Thiers has assumed the direction of the Chemistry Laboratories of the Peter Bent Brigham Hospital. Dr. Bartholomay, who has been appointed Assistant Professor of Mathematical Biology in the Harvard School of Public Health, informally functions as adviser in mathematics to the Department of Medicine of the Peter Bent Brigham Hospital

For the fourth year the laboratory has been requested to participate in the teaching of biophysics at Massachusetts Institute of Technology. Doctors Valles and Thiers take part in the formal lecture course. Laboratory sessions are given at the Peter Bent Brigham Hospital. Mr Donald Plocke, a graduate student in biophysics at the Massachusetts Institute of Technology, has undertaken his doctorate thesis in the laboratory under this program.

Mr. Thomas L. Coombs, a graduate of the University of London, is registered there as a candidate for the Ph.D. under that university's overseas program and has been granted permission to complete his thesis in the Biophysics Research Laboratory.

The grant from the Armed Services allowed the establishment of the laboratory and the accomplishment of the objectives detailed above. From the viewpoint of the laboratory the collaboration with the Armed Services was most successful. The administrative quarters could not possibly have been more cooperative or helpful. The administrative demands were kept at an absolute minimum, and the atmosphere created was as favorable to the pursuit of knowledge as any scientist could hope for The entire staff is deeply appreciative of the interest and consideration which it has been shown at every turn, and it is regretted sincerely by one and all that this contract, like all good things, must come to an end. The association has engendered for all concerned a most receptive attitude toward whatever the future may bring in the way of cooperation with the Armed Services.

PERSONNEL

Present Staff Members

Bert L. Valles, M.D.
Frederic L. Hoch, M.D.
Warren E.C. Wacker, M.D.
Ralph E. Thiers, Ph.D.
Anthony F. Bartholomay, M.D.
Carl A. Price, Ph.D.
Keiichiro Fuwa, Ph.D.
Jeremias Maegi, M.D.
Philip J. Snodgrass, M.D.
Edward S. Reynolds, M.D.
Thomas L. Coombs, M.S.
[redacted] Fiscina, M.S.
Raoul DeNapoli, M.D.

Past Staff Members and Their Present Professional Activities

Stanley J. Adelstein, M.D., Ph.D. - President, Peter Bent Brigham Hospital
Marvin Margoshes, Ph.D. - Spectroscopy Division, National Bureau of Standards, Washington, D.C.
Robert Chaffee, Ph.D. - Research work - U.S. Army Air Force Base, Randolph Field
Reinosuke Hara, Ph.D. - Atomic Energy Commission, Japan
H. Richard Tyler, M.D. - Neurologist, Peter Bent Brigham Hospital
Hans Cohnheim, M.D. - Professor of Pathology, Institute for Tropical Research and University of Hamburg, Germany

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